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14. ABSTRACT
In this program we have tested the concept that carbon nitride nanowires (CNWs)-conjugated to Herceptin can selectively target and photo-thermally ablate HER2-positive breast cancer tissues using penetrating near infra-red radiation (1.06 microns). Our initial results in cell culture suggest that CNWs are far superior transducers of radiation to the SWNTs or Ag nanoshells due to their metallic nature and aspect ratio. Initial animal studies have begun.

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Our objectives were:

Objective 1: Development of Herceptin-CNWs in cell culture: Using our experience conjugation chemistries on fullerenes, we will construct CNW conjugates and demonstrate that their photo-ablative properties are significantly superior to those referenced above.

Materials and methods

<u>MWNT growth and characterization</u>
CN_x-MWNT were produced by carbon vapour deposition (CVD) as described previously (Liu, Czerw, & Carroll 2005). As precursors the catalyst ferrocene ($Fe(C_5H_5)_2$) and the carbon-nitrogen source pyridine (C_5H_5N) were used together with hydrogen as carrier gas. X-ray photoelectron spectroscopy (XPS) was used to determine the overall nitrogen concentration in the CN_x-MWNT, which was found to be 1% - 2%. In order to produce CN_x-MWNT of various lengths, the nanotubes were ultrasonicated in a mixture of concentrated sulfuric and nitric acid (3:1) for 7, 24 and 60 hours. After extensive washing and drying, the purity and length distribution of all samples were examined using a Philips 400 transmission electron microscope (TEM) operating at 80 keV. All samples showed almost no catalytic or carbonaceous particles on the MWNT surface; this is indicative of highly purified nanotube material. The length distribution of the samples sonicated for 7, 24 and 60 hours is shown in (Figure 1). Their average length was found to 1100 nm (7 hours), 700 nm (24 hours) and 300 nm (60 hours). Average widths were 15 nm, 15 nm and 13 nm, respectively. Since in the present experiments the ratio of nanotubes to cells was varied, all experiments were normalized to nanotubes per cell. In order to count the nanotubes in each of the samples, a simple TEM based method was devised based on placing a known volume of dispersed CN_x-MWNT on a TEM grid, evaporating the aqueous buffer, and counting the number of nanotubes on the grid. Before use, CN_x-MWNT were homogeneously suspended in Hepes Buffered Saline (HBS) by sonication in a Bronson 3510 sonicator for approximately one hour.

<u>Cell culture and viability</u>
CRL 1932 cancer cells were obtained from the American Type Culture Collection and cultured at 37 °C in a humidified chamber containing 5% CO_2 in Mc Coy's 5A media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were seeded at approximately 1.5×10^4 cells/well in 48-well tissue culture plates. 0.1 ml of (CN_x-MWNT) suspended in HBS were mixed with 0.9 ml CRL 1932 cancer cells in McCoy's 5 A modified media. The number of viable cells per ml of cell suspension was determined using a hemocytometer following staining with trypan blue. The dishes were incubated at 37 °C overnight.

The ratio of nanotubes to cells was varied in order to find the threshold for cell death. The concentration of nanotubes in the original suspension was determined by counting the number of nanotubes following deposition of a known volume of nanotube suspension onto a TEM grid as described above. Using this method and the conventional cell counting method described above, calculated ratios of nanotubes/cell of 1000:1, 100:1 and 1:1 were used. Where necessary, nanotubes suspensions were diluted in HBS. The concentrations for the 1000:1 samples were 0.083 mg/ml (1100 nm MWNT), 0.027 mg/ml (700 nm MWNT) and 0.005 mg/ml (300 nm MWNT). Furthermore, for each experiment a control sample of HBS without nanotubes was prepared. After incubation with or without nanotubes for 24 hours, the cells were irradiated with a NIR quasi-CW-YAG laser operating at a wavelength of 1064 nm and laser power of 3 W/cm^2. Each well was

exposed to the laser for 4 minutes. Cell viability was assessed by trypan blue exclusion or in some cases by crystal violet staining. Temperature was measured using a thin thermocouple wire, attached to a Fluke thermometer, suspended in the growth media so that increases in the media temperature could be determined as a function of lasing time and nanotube length.

Results and discussion

Nanotube length and lack of inherent toxicity
Our initial objective was to determine whether CN_x MWNT exhibit inherent toxicity in the absence of IR treatment. To test this, we first used CN_x MWNT of a fixed average length of 1100 nm. These were produced by limited acid treatment, as shown in Figure 1A. Cells were mixed with CN_x MWNT at a calculated
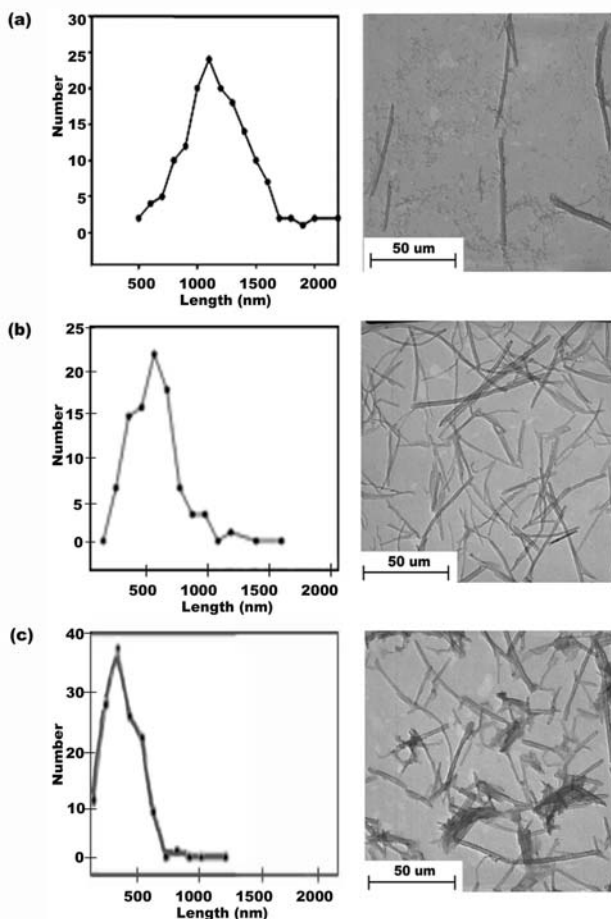
Figure 1: Sonication reduces mean length of CN_x-MWNT. CN_x-MWNT were sonicated in acid as described in Materials and Methods for 7 hours (a), 24 hours (b), and 60 hours (c). Average length was assessed by TEM.

ratio of 1000 nanotubes to one cell and effects on viability assessed over 48 hrs. As shown in Figure 2, CN_x MWNT by themselves had no discernible effect on cell viability over this timeframe. Thus, CN_x MWNT are not inherently cytotoxic, in accordance with the recent literature (Worle-Knirsch, Pulskamp, & Krug 2006).
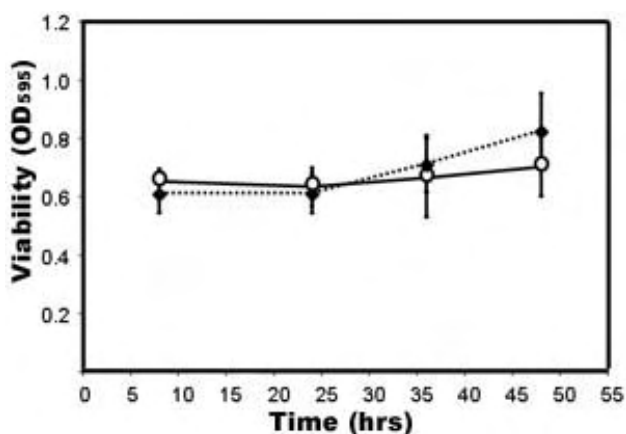

Figure 2. CN_x-MWNT do not inhibit cell growth. 1.5 x 10^4 cells/well CRL 1932 cancer cells were plated with 1.5 x 10^7 1100 nm nanotubes in 35 mm wells. Control samples contained no MWNT. Viability was measured using a crystal violet absorption assay at 595nm after 8, 12, 24, 36 and 48 hours of culture. Means and standard deviations of triplicate determinations are shown. ----●----- is control, ——○—— is 1000 nanotubes per cell.

Heat generated by CN_x-MWNT
We next assessed the degree to which irradiation of CN_x MWNT with NIR could induce an increase in temperature. These experiments were performed with 700 nm MWNT. Nanotubes were diluted in media and irradiated for various time at different concentrations. As shown in Figure 3, an increase in temperature was attained when nanotubes were irradiated with NIR in aqueous media. The concentrations used were 1000 x 10^5 nanotubes per unit volume (marked 100 %), 500 x 10^5 nanotubes per unit volume (marked 50%), 250 x 10^5 nanotubes per unit volume (marked 25%), and finally 10 x 10^5 nanotubes per unit volume (marked 10%). We note that the heating of media drops as the

concentration of nanotubes drops. We address this point below.

## Cell viability following CN$_x$ MWNT induced photothermal effect
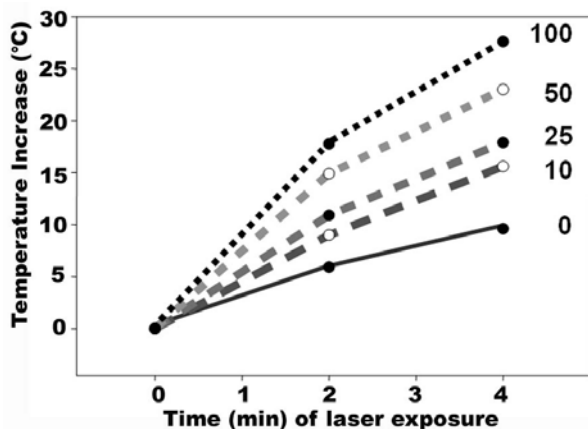
Figure 3. Effect of nanotube concentration on heating. Concentrations of nanotubes used start at 1000 x 105 nanotubes/unit volume (marked as 100%). The concentration was then diluted to 500 x 105, 250 x 105, and 100 x 105 nanotubes/unit volume, (marked 50%, 25% and 10% respectively). Starting temperature was approximately 23oC.

To test whether a decrease in viability could be induced by combined treatment with 1100 nm CN$_x$ MWNT and IR, cells were incubated with various concentrations of CN$_x$ MWNT, allowed to adhere overnight, and then exposed to 3W/cm^2 IR light for 4 minutes. Cells were incubated with CN$_x$ MWNT at nanotube:cell ratios of 1:1, 1:100, and 1:1000. Control cells were incubated with CN$_x$ MWNT, but were not treated with laser, or were treated with laser in the absence of nanotubes. Viability was assessed in all cultures. As shown in Figure 4A, in the absence of nanotubes, treatment with NIR had no effect on viability. Thus, cells have a high transparency to NIR light, and neither exposure to NIR nor exposure to nanotubes alone is sufficient to induce cell death. However when cells were incubated with CN$_x$ MWNT and subsequently exposed to NIR, there was a dramatic dose-dependent decrease in viability, with over 90% cell death at the highest dose tested (Figure 4A). The greatest decrease in viability

Figure 4. Effect of 1100 nm CNx-MWNT. (a) 1.5 x 104 cells/well were mixed with 0.1 ml of 1100 nm CNx-MWNT in HBS at various concentrations in 10 mm wells. Cells were allowed to attach overnight and exposed to YAG laser for 4 minutes. Untreated controls

are also shown. Viability was assessed by trypan blue exclusion. Means and standard deviations of 6 replicate cultures are shown. Similar results were obtained in six independent experiments. (b) Change in temperature of CRL 1932 cancer cells plated with various concentrations of N-doped MWNT after exposure to YAG laser for 4 minutes. Initial temperature was approximately 23°C. Maximum temperature change of 23.5 ± 0.3 ^{o}C in 1000x N-doped MWNT sample. Abbreviations for this and subsequent figures are: NT (nanotube), L (laser), NL (no laser).
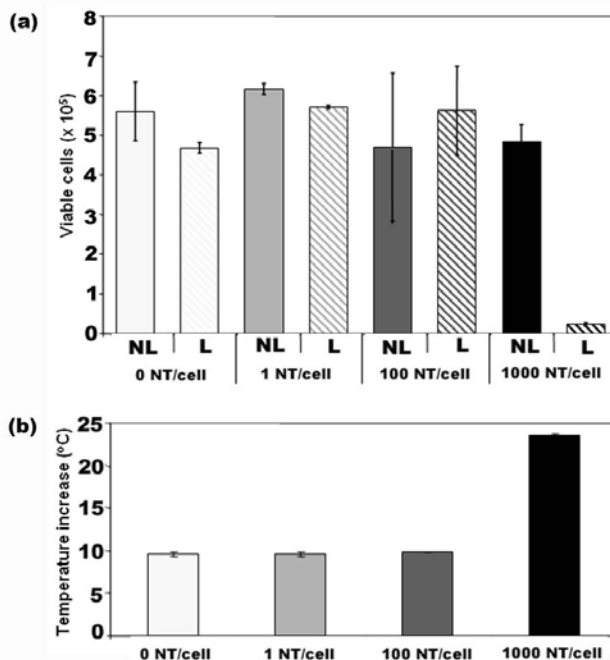
was associated with the greatest increase in temperature: the average number of viable cells in 1000x sample decreased by 96.25% after exposure to laser, where a maximum temperature of 57.7 ± 1.5 ^{o}C was attained.

We next cultured cells with CN$_x$ MWNT at cell:nanotube ratios of 1:1, 1:100, and 1:1000 (designated as 1x, 100x and 1000x, respectively), irradiated for a fixed time of 4 minutes each, and tested whether temperature increases occurred under these conditions. As can be seen in Figure 4B, there was a slight increase in temperature in the control, 1x and 100x samples. The highest temperature change was found in the 1000x sample, which reached a maximum temperature of 57.7 ± 1.5 ^{o}C.

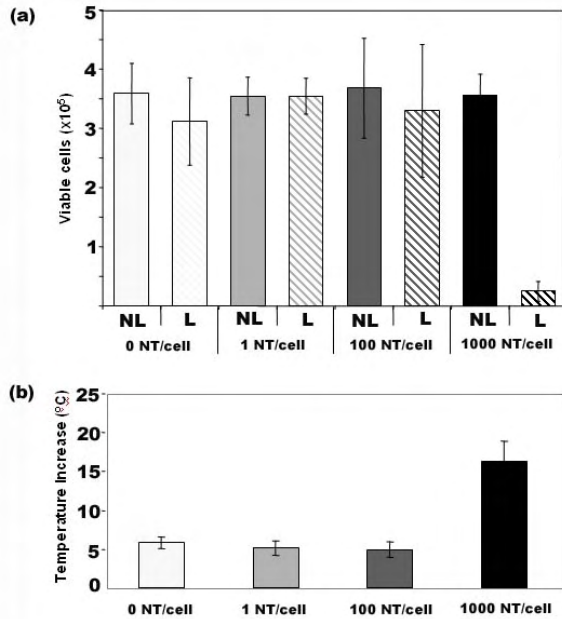## Control of CN$_x$ MWNT nanotube properties by length modification

In order to predict and control the properties of CN_x-MWNT nanotubes as heating agents, it was important to determine whether their behavior could be modeled by classic antenna theory. One prediction of this theory is that optical coupling should occur at nanotube lengths that are at least half the wavelength of the incident light. In order to assess whether the behaviour of CN_x-MWNT obeyed this prediction, we prepared nanotubes of different lengths and compared their activities as thermal coupling and cytotoxic agents.

As shown in Figure 1B, by increasing the length of acid treatment from 7 hrs (used to produce 1100 nm tubes) to 24 hrs, nanotubes of 700 nm average length were produced. Acid treatment for 60 hrs further reduced average length of CN_x-MWNT nanotubes to 330 nm as measured by TEM (Figure 1C).
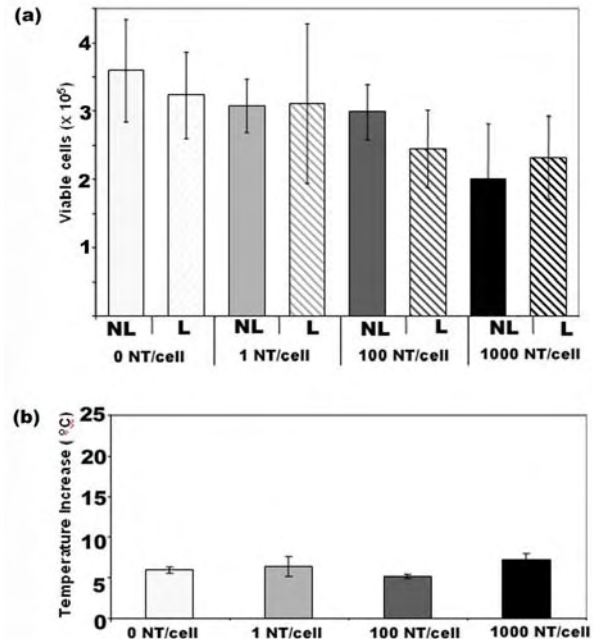
These nanotube preparations were incubated with cultured cancer cells, and their effects compared to those of 1100 nm CN_x-MWNT.

Figure 5. Effect of 700 nm CN_x-MWNT **(a)** 1.5 x 10^4 cells/well were mixed with 0.1 ml of 700 nm CN_x-MWNT in HBS at various concentrations in 10 mm wells. Cells were allowed to attach overnight and exposed to YAG laser for 4 minutes. Untreated controls

are also shown. Viability was assessed by trypan blue exclusion. Means and standard deviations of 6 replicate cultures are shown. Similar results were obtained in seven independent experiments. (b) Change in temperature of CRL 1932 cancer cells plated with various concentrations of N-doped MWNT after exposure to YAG laser for 4 minutes. Initial temperature was approximately 23°C. Maximum temperature change of 16.35 ± 2.5 °C in 1000x N-doped MWNT sample.

As seen in Figure 5A, similar to the results obtained with 1100 nm CN_x-MWNT, cells incubated with 700 nm N-doped MWNT in the absence of laser exposure maintained viability. Laser treatment by itself (in the absence of nanotubes) was also non-toxic (Figure 5A). However the combination of nanotubes and laser treatment again induced a dose-dependent cell death and temperature increase in the culture: There was little increase in temperature or effect on viability in cultures treated with CN_x-MWNT at nanotube:cell ratios of 1:1 or 100:1. However, the average number of viable cells in cells treated with nanotubes at a ratio of 1000:1 decreased by 91.94% after exposure to laser with a maximum temperature of 50.65 ± 5.7 °C (Figure 5B). Although the extent of cell death and maximal temperature increase were slightly less than those observed with 1100 nm nanotubes, these results indicate that CN_x-MWNT of 700 nm length are also efficient at thermal coupling.

Fig. 6. Effect of 330 nm CN_x-MWNT (a) 1.5 x 10^4 cells/well were mixed with 0.1 ml of 330 nm CN_x-MWNT in HBS at various concentrations in 10 mm wells. Cells were allowed to attach overnight and exposed to YAG laser for 4 minutes. Untreated controls are also shown. Viability was assessed by trypan blue exclusion. Means and standard deviations of 6 replicate cultures are shown. Similar results were obtained in two independent experiments. (b) Change in temperature of CRL 1932 cancer cells plated with various concentrations of N-doped MWNT after exposure to YAG laser for 4 minutes. Initial temperature was approximately 23°C. Maximum temperature change of 7.2 ± 0.8 ^{o}C in 1000x N-doped MWNT samples.

Finally, the effect of CN_x-MWNT of 330 nm average length were assessed. Like the 1100 and 700 nm nanotube preparations, these exhibited no toxicity in the absence of laser (Figure 6A). However, in contrast to results obtained with 1100 nm and 700 nm nanotubes, no cell death was seen at any concentration of these nanotubes. Maximal increase in temperature was correspondingly decreased: the maximum temperature reached in 1000x sample was 41.78 ± 2.85 ^{o}C (Figure 6B). This temperature was only slightly above the normal incubation temperature of 37°, and was thus insufficient to cause cell death. Since 300 nm CN_x-MWNT are less than of half the wavelength of light used to activate the nanotubes (1064 nm), these results are consistent with the prediction that these nanotubes should not exhibit effective coupling to the laser. A comparison of all three nanotube lengths and corresponding cell survival is shown in figure 7.

Nanotube NIR coupling and heat transfer
The dependence of heating and cell death on nanotube length also suggests that CN_x-MWNT behave as anticipated by classic antenna theory, which predicts that for nanotubes to couple effectively, they must be at least as long as one half the wavelength of the incident radiation (Hanson 2005;J.E.Riggs et al. 2000;J.E.Riggs, D.B.Walker, & Y.P.Sun 2001;Y.Wang et al. 2004). These wavelengths allow the antenna to become an electrical dipole from incident radiation and absorb light efficiently. Consistent with this prediction, 700 nm and 1100nm nanotubes delivered substantial heat to the cellular media when exposed to 1064 nm light, whereas 300 nm nanotubes coupled poorly to the radiation field and consequently did not absorb enough light to raise the media temperature.

Thermodynamic calorimetrics can be used to estimate how far these nanotubes transfer the heat generated within them into the surrounding liquid. The calorimetric equation (1) that must be satisfied on an instantaneous level is:

(1) $M_{NT} C_{NT} \Delta T_{NT}^i = M_{water} C_{water} \Delta T_{water}^f + M_{NT} C_{NT} \Delta T_{NT}^f$

where the M's are the masses, the C's are the heat capacities, and the ΔT's are the changes in temperature.(Charles Kittel & Herbert Kroemer 1980) This states that the amount of heat (Q) lost from the hot nanotube can be accounted for in the final temperature of the water (cellular milieu) and the nanotube combined. Because the system is not closed, this is only a first approximation of the experimental system. Further, bundling of the nanotubes is not accounted for in these experiments. However, this can provide us with an expectation of the simple best case scenario. To estimate the zone of heat transfer, we can ignore the second term, because it is small compared to the total mass of the water, and rearrange equation (1) to obtain equation (2):

(2) $R_{ZONK} = \{\dfrac{\frac{3}{4}\pi(M_{NT}C_{NT}100^{\circ}C)}{\rho_{water}C_{water}50^{\circ}C}\}^{1/3}$.

R_{ZONK} is the "zone of nanotube killing" (ZONK), the radius from the surface of the nanotube that defines a volume in which the temperature has reached a minimum of 50°C. ρ_{water} is the density of water. If we choose 100 ^{o}C as an initial temperature of the nanotube, then the zone of cell killing around a single N-doped MWNT can be estimated as follows: C_{NT} ~ 3000, C_{water} ~ 1000 (Hepplestone et al.), and the radius of a MWNT of about 10^{-8} m. This gives a ZONK radius of roughly 10^{-7} m. If we allow for nanotube temperatures to be 1000 ^{o}C, then the radius extends to approximately one micron. These calculations demonstrate that in the best case, a single nanotube can effectively heat volumes immediately adjacent to it.

The combined contributions of nanotube mass and number may explain why cell killing required high nanotube:cell ratios. In these studies, we have used a relatively low radiation dose (720J) in order to stay within a range that would not damage dermal layers should this be applied as a therapeutic. At these radiation doses, the heat is relatively well localized to one or two ZONK radii for an individual nanotube. As the number of nanotubes is increased, for a constant irradiation power, we expect the heat they can transfer will increase as the cube root of their mass. However, the number of antenna used increases the heat transfer linearly. So, from these simple considerations, we

expect that larger nanotube masses will enhance the total volume of killing, but not nearly as rapidly as increasing their numbers. This is seen in Figure 3 where the increase in maximum temperature is nearly linear with concentration for a given time. The solutions used in Figure 3 are well dispersed and therefore we expect clumping to have little effect.

Recently, nanomaterials have been applied to thermal ablation of tumors. These include gold nanoshells,(Hirsch, Stafford, Bankson, Sershen, Rivera, Price, Hazle, Halas, & West 2003) and single walled nanotubes SWNT.(Kam, O'Connell, Wisdom, & Dai 2005) All of these materials are in early developmental stages. CN_x-MWNT have some theoretical and practical advantages as a nanomaterials. First, as discussed above, MWNT are excellent antenna for electromagnetic energy, and will absorb approximately three times the light as SWNT. This is important because within the transmission window of 700 nm to 1000 nm of human tissues, light is attenuated by skin and subcutaneous tissues. Since near IR irradiation may be applied through the skin to kill embedded cancers, the decreased light intensities required to heat with MWNT may reduce damage to dermal layers, particularly when the cancer lies at the deeper end of the NIR penetration range (2-4 cm). A second advantage of MWNT is that they have broad band width compared to the specific resonance absorptions of SWNT and nanoshells, allowing them to be activated by broader band widths of electromagnetic radiation. A third distinction is that MWNT do not require cell uptake, since we have shown that if they are within the R_{zonk} kiliing radius, they are sufficiently close to impart enough thermal energy for cell damage.

Our experiments demonstrate that N-doped MWNT's can act as highly efficient heat transduction molecules in the extracellular environment. Further, we have shown that heat transfer and subsequent cell killing can be achieved extracellularly with relatively low total radiation doses of 1064 nm light. We have suggested that both the mass of the N-doped MWNTs and their length play an important role in radiation transduction and heat transfer. Since previous studies have shown that such long nanotubes cannot be taken up by cells in general, the fact that such effective cell killing was achieved outside of the cell demonstrates that photo-thermal techniques involving "nano-antennae" are not limited to those materials that can be endocytosed, which allows for a wide variety of therapeutic possibilities for the delivery of nanotubes to tissues. Additionally, the radiation wavelengths and doses used in this study suggest that such approaches might be feasible for the treatment of cancer with externally applied electromagnetic radiation.

Conclusion

The overall goal of our experiments was to test whether CN_x MWNT could be used as effective heat transducers for photoablation of cultured cancer cells. We hypothesized that these nanotubes would be non-toxic *per se*, but could be activated to release heat by exposure to near-infrared radiation, and that this in turn would lead to cell death. We further hypothesized that effects of nanotubes would be dependent on their length. We observed that nanotube lengths of 1100 and 700 nm, but not 300 nm, coupled with the radiation and caused significant increases in temperature and cell death. The low power levels (3 watts) necessary to effectively kill cells suggests that the efficiency of NIR coupling to the long nanotubes is high. In summary, these results demonstrate that CN_x-MWNT are versatile and effective heat-delivery and cytotoxic agents. The ability to control their properties through length and other modifications may facilitate the ultimate clinical application of CN_x-MWNT in the treatment of cancer.

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Objective 2: Targeting and ablation of breast cancer cells in cell culture and mouse models, with the Herceptin-CNWs: We will test whether Herceptin conjugated to the CNWs retains the specificity to HER2-positive cells in tissue. Further we will demonstrate that the conjugated nanotubes photothermally-ablate Herceptin positive breast cancer cells orthotopically implanted in athymic nude mice.

Experiments with animals have only just begun. Nude mouse models are in place. First experiments will use non-targeted CNx tubes. This is being carried out by direct injection of tumor zenographs in the rear quarters of the mice.

These results will be described in the next report.